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## CHOLERA.\*

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### SIMPLE METHODS OF BACTERIOLOGICAL DIAGNOSIS.

In the examination of many specimens of feces for the presence of cholera vibrios, labor and time spent must be reduced to a minimum. This is especially evident in the examination of suspected carriers where it may be necessary to handle hundreds of cases a day. With such an amount of material even the best equipped laboratory would be crippled in attempting to carry out any but the simplest methods, especially in the face of an emergency. For reasons of expediency, the following simple method has been adopted after trying different methods suggested by other workers.

*Collection of material.*—Feces should be obtained whenever feasible and should reach the laboratory promptly, though if a large number of persons must be examined the collection of feces is almost impossible. The procedure adopted by Dr. Doty of taking rectal swabs is simpler and seems to be fairly reliable. These swabs are the ordinary wire, cotton-tipped swabs, such as are used in diphtheria work. They should be moistened with sterile peptone solution to facilitate introduction and prevent drying during transit, which might cause the death of the vibrios if there is any delay in reaching the laboratory. We have distributed the swabs with several drops of peptone solution in the bottom of the tube, against which the cotton rests.

*Media.*—Three media have been used, viz., Dunham's peptone water, Dieudonné's<sup>†</sup> blood agar, and alkaline 3 per cent agar. Not all are necessary in routine work.

#### *Outline of method.*—

Feces.—Direct examination.

Peptone water.—Incubate eight to 12 hours. Examine smears from surface growth. Examine those showing vibrios, in hanging

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<sup>†</sup> *Centralbl. f. Bakt.*, I, Orig., 1909, 50, p. 107.

drop, with and without agglutinating serum, making the hanging drops from the surface growth. If vibrios are too few in number for this or if smears are negative subinoculate into second peptone tubes.

Peptone water (subculture).—Incubate eight to 12 hours. Examine as above. Those showing no vibrios or too few to see in the hanging drop are considered negative.

*Direct examination.*—This is of no value except in acute cases. If a large number of typical and extremely motile vibrios are present, the case may be considered positive. The difficulty is that occasionally stools from cases other than cholera show rather numerous vibrios.

*Peptone water.*—There is nothing to be gained in examining hanging drops from tubes in which no vibrios are revealed by smear. Further, it is a waste of time to examine the smear too long, for if the tubes contain cholera vibrios sufficient for diagnosis they are very quickly found. On the other hand, if they are few in number, a second enrichment becomes necessary.

The surface growth in mixed cultures containing cholera is so nearly pure that on the addition of agglutinating serum the prompt clumping and loss of motility is sufficiently evident to be diagnostic. This application was suggested by Pollaci.<sup>1</sup> It is seldom that other vibrios enrich to this extent. If they do they can be excluded by the absence of any effect of the serum. When they are fewer in number, their motility may be like that of the cholera vibrio, which motility makes them evident even in hanging drops of mixed cultures. If the serum has no influence on this motility the vibrio can be excluded as not being cholera.

*Subculture: peptone water.*—A subculture in peptone water is necessary, as in four instances we have found cholera vibrios in subcultures where the first series of tubes were negative. The feces in these cases were not from cases showing clinical evidences of cholera. In the examination of acute cases no such necessity of further enrichment is likely to arise. In the examination of suspected carriers, however, an appreciable number of positive cases would be overlooked without a second enrichment. This

<sup>1</sup> *Deutsch. med. Wchnschr.*, 1911, 37, p. 354.

second enrichment helps to exclude the vibrios other than cholera. Some will have died out, others will not have enriched beyond the amount present in the first tube; these are not cholera. All vibrios which have enriched are then differentiated with agglutinating serum.

In examining for carriers the examination of the first peptone tubes may be dispensed with altogether. No fear need be entertained that cholera vibrios will be lost; as a matter of fact, they will enrich still further in the second tube. This delays the findings in some instances but becomes necessary where the number of examinations is large. In transferring the cultures, the first peptone tubes should be inspected, as positive tubes show a characteristic surface cloud. Any tubes showing this should be examined at once.

This method requires no elaborate equipment and allows the examination of large numbers of cases with the least expenditure of time. If selective media are used, such as the Dieudonné or the modification described in the next section, they should be used in duplicate with the peptone tubes. The great advantage of this method is its applicability to emergency work.

#### SELECTIVE MEDIA. A NEW AND SIMPLE MODIFICATION.

When Dieudonné prepared his alkaline blood agar, he was the first successfully to apply a fact known for years, viz., that the cholera vibrio could grow in the presence of alkali sufficient to inhibit other fecal bacteria. Deycke had attempted the use of alkaline albuminates in 1893 but without uniform results.

The formula of Dieudonné's medium as now in use is as follows:

- |    |                       |        |
|----|-----------------------|--------|
| A. | Defibrinated blood    | } a.a. |
|    | Normal sodium hydrate |        |

Mix thoroughly and steam in the Arnold sterilizer for three-quarters of an hour.

B. Meat peptone agar, neutral to litmus, containing 3 per cent of agar.

Mix A, 30 parts, and B, 70 parts, while hot, and pour thick plates. The plates are then left open for 20 minutes to dry. They

are then placed in an oven at 50° to 60° C. for 20 to 30 minutes and finally left half-open in the incubator overnight. If used before this treatment cholera itself will not grow on the plates.

Since Dieudonné others have added facts which tend to simplify the preparations of the medium. Pergola<sup>1</sup> found that the meat was not necessary in the agar, and that if this were left out the agar could be used without correcting the reaction. Recently Pilon<sup>2</sup> has substituted a 12 per cent crystalline sodium carbonate solution for the sodium hydrate. The plates can then be used at once; in fact, the blood mixture need not be heated if the plates are used immediately.

During our work with the Dieudonné medium it seemed to us susceptible of improvement in two particulars: first, if the medium could be rendered transparent; second, if a more easily obtainable material could be substituted for the blood. Where cholera examinations are of infrequent occurrence, or where blood is not easily obtained, such a change would be extremely important.

Experiments were then undertaken substituting eggs for the blood. Various mixtures were tried of the white alone, giving a transparent medium, and the whole egg, which gives a translucent medium. The following combinations were found satisfactory, in that they showed the requisite amount of restraint of the fecal bacteria with free growth of the cholera vibrio.

#### Egg-white medium.

- |                                                                            |   |                                                                      |
|----------------------------------------------------------------------------|---|----------------------------------------------------------------------|
| A. White of egg and water, a.a.<br>Sodium carbonate cryst. 12<br>per cent. | } | Mix in equal parts, steam<br>in Arnold sterilizer for<br>20 minutes. |
| B. Meat peptone 3 per cent agar, neutral to litmus.                        |   |                                                                      |

#### Whole-egg medium.

- |                                                                         |   |                                              |
|-------------------------------------------------------------------------|---|----------------------------------------------|
| A. Whole egg and water a.a.<br>Sodium carbonate 12 to<br>13.5 per cent. | } | Mix in equal parts, steam<br>for 20 minutes. |
| B. Meat-free agar, viz., peptone, salt, and 3 per cent agar.            |   |                                              |

In either case mix A, 30 parts, and B, 70 parts, while the agar is boiling hot. Pour medium thick plates, allow them to stand

<sup>1</sup> *Centralbl. f. Bakt.*, I, Orig., 1911, 59, p. 83.

<sup>2</sup> *Ibid.*, 1911, 60, p. 330.

open for 20 to 30 minutes to dry, and then inoculate by surface streaking.

In making the egg mixtures, the water and egg must be thoroughly mixed, the alkali added and again well shaken. Filtration through a thin layer of cotton may be employed to remove any of the thicker parts of the egg.

Of the two formulae, the whole egg gives the more vigorous growth of vibrios, requires no meat in the agar, and is more economical. The egg white, on the other hand, requires a meat agar, the growth is not as vigorous, and crystals frequently form, especially along the inoculation streaks. Its only advantage is its transparency. For general use, therefore, the whole-egg mixture is better. Comparing the whole-egg medium with the blood medium of Dieudonné, the latter is somewhat more selective; that is, it seems to restrain the common fecal bacteria somewhat more. This, however, is more than offset by the fact that should other bacteria grow the cholera colonies on the egg medium are distinctive. Examined by transmitted light they have the appearance of being deep in the agar and have a peculiar hazy look, due to a halo about the colony. Where the growth is very vigorous this halo is surrounded by a zone of clearing. In practice, fishing from this type of colony has yielded cholera and from this type alone. The only exception is in the case of cholera-like vibrios, their colonies being identical. The plates keep when stored on ice for a limited time. The egg alkali mixture seems less permanent. This is unimportant compared with the more permanent Dieudonné's blood mixture, as the egg mixture can be prepared so quickly.

Because of the ease of preparation and the prompt availability of the ingredients this medium is an improvement, especially for those who are called upon to examine cholera-suspected stools at infrequent intervals. When one considers that 10 eggs is sufficient for over 300 plates the medium is economical even where large numbers of examinations are being made. Furthermore, it is an improvement for the reason that cholera grows in distinctive colonies, allowing quick selection for smears and agglutination.

## CHOLERA-LIKE VIBRIOS.

The vibrios other than cholera found in stools are of little practical importance. When, however, the present trouble arose there was no cholera agglutinating serum available for immediate use. Under these circumstances the detention of a ship was necessary because of the presence of a cholera-like vibrio in one case. This is an emergency that is likely to arise at any time in a country where cholera examinations have not been required for many years.

Because of this we have tested the various vibrio strains we have isolated, to see if there were any biological peculiarities which would quickly exclude those not cholera. This has yielded some results. Some of the vibrios can be excluded because of the marked tenacious pellicle they form on peptone, or the dry adherent growth on agar. Of 50 vibrios other than cholera, 43 did not give the cholera-red reaction.<sup>1</sup> Of the seven that did, two did not produce acid, and four produced gas in litmus-glucose peptone, only one produced acid like cholera, but this strain formed a tough pellicle and was adherent on agar. These differences were evident in less than 24 hours' incubation.

If, then, a colony be fished and inoculated into peptone water, glucose media, and agar, many, if not the majority, of vibrios other than cholera could be quickly excluded.

The ability to liquefy Loeffler's blood serum and gelatin and the fermentation of saccharose and lactose as well as milk were tried. The acid change in lactose, milk, and the liquefaction of gelatin and Loeffler as exhibited by cholera is too slow to be of practical use. The fermentation of saccharose is more prompt. About half of the other vibrios ferment this sugar, some with the production of gas.

A few vibrios we have found to be only slightly motile. When mixed cultures containing vibrios are subinoculated in peptone water, they do not show the same ability to enrich as shown by cholera. Many enrich to a limited degree and then remain con-

<sup>1</sup> Care should be used in determining this, as in several instances we got a reaction with peptone water to which sodium hydrate had been added to increase the alkalinity, not otherwise. Whether this was due to traces of nitrate we cannot say.

stant. Others enrich slightly and die out in the third tube of peptone. This may be considered evidence that they are not cholera.

#### HEMOLYTIC ACTION OF CHOLERA AND ALLIED VIBRIOS.

Because of the number of varied reports that have appeared on the subject of hemolysis by cholera and other vibrios, we have tested 50 cholera and 50 non-cholera strains for their hemolytic properties. These were recently isolated and included one culture from the Philippines and two from Italy. One old culture isolated by Buxton in 1893 and one El Tor strain were also tested. This latter strain is one of the six isolated in 1905 by Gottshlich at El Tor from the cadavers of pilgrims to Mecca. These pilgrims had died with diarrheal symptoms, but had shown no clinical evidences of cholera. The El Tor strains agglutinated like true cholera, but were strongly hemolytic. The hemolytic power was considered by some, especially Kraus and Ruffer, to be sufficient evidence for regarding these vibrios as not true cholera. This was based on Kraus' statement that the hemolytic power was not a property of true cholera, but was only found in allied types.

Our results were as follows:

|                                     |                     |
|-------------------------------------|---------------------|
| Thirteen non-cholera vibrios.....   | weakly hemolytic.   |
| Four non-cholera vibrios.....       | strongly hemolytic. |
| El Tor.....                         | strongly hemolytic. |
| Cholera, Buxton. <sup>1</sup> ..... | strongly hemolytic. |

The following technic was employed: to 1 c.c. of a suspension of washed sheep, goat, or horse corpuscles, was added .25 c.c. of a week-old broth culture. Readings were made after two and three hours' incubation. The tubes were then shaken and put in ice for 24 hours, when final readings were taken.

Our results with the cholera vibrios agree with those of Mühlens, von Raven, Neufeld, and Haendel, and others who have found that laboratory cultures of true cholera may possess or acquire hemolytic power.

<sup>1</sup>This strain showed cultural variations. It did not liquefy Loeffler's blood serum and was extremely slow to liquefy gelatin. We could not test its agglutinability, as it made a poor suspension which quickly flaked out. In rabbits, however, it produced agglutinins for true cholera.

On the other hand, Mühlens and von Raven found that three rather recently isolated strains possessed hemolytic powers. Baerthlein<sup>1</sup> also found some newly isolated strains strongly or weakly hemolytic, depending upon the technic used. When he inoculated corpuscle suspensions from agar slants and incubated 24 hours or longer, he found one strain strongly, and others irregularly, hemolytic. When he used a five-day broth culture, the same strain showed comparatively prompt hemolysis. All, however, showed varying degrees of hemolysis at 48 to 72 hours. With blood agar plates again only the one strain showed hemolysis.

Although the technic of different observers has differed so greatly as to make it difficult to correlate the results, there can be no doubt that there is great variability in the hemolytic powers on successive tests even of the El Tor cultures. Freshly isolated cholera cultures do not possess strong hemolytic powers, though this property may be acquired after artificial cultivation for longer or shorter periods of time. In a few cases freshly isolated strains have been strongly hemolytic or have shown traces of this capacity. This capacity may not be as well developed as in some non-cholera vibrios, but it cannot be considered of sufficient importance to justify the separation of strains biologically true cholera but hemolytic, from non-hemolytic strains. If a strain, El Tor or other vibrio, agglutinates in a cholera-immune serum, it must be considered cholera, whether it hemolyzes or not. The fact that the El Tor strains were isolated from pilgrims who must have become infected months previous to their death, that is, were carriers for an unusually long period, and who, during the pilgrimage, were not the source of cases of cholera, is none the less remarkable than that each strain isolated should be the same in possessing hemolytic properties.

<sup>1</sup> *Arb. a.d.k. Gsundheitsmte.*, 1911, 36, p. 446.